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ISOLATION AND CHARACTERIZATION OF ACID-SOLUBILIZED COLLAGEN FROM THE SKIN OF FRESHWATER SNAKEHEAD FISH *CHANNA STRIATUS"*

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ABSTRACT

Acid-Solubilized Collagen was extracted from the skin of freshwater snakehead fish *Channa striatus* and characterized (SDS-PAGE, Amino acid, FT-IR study, Solubility). The SDS-PAGE patterns were identified to be the type-I collagen with major components of α1, α2 and β chains. The characterization of amino acid showed that glycine occupies more when compared to other acids followed by alanine, proline, hydroxyproline and glutamine. The FT-IR study shows the presence of the all the three amides (Amide I, II and III) with their respective frequencies.

KEYWORDS: Channa striatus, snakehead fish, Acid solubilized collagen, type – I collagen.

INTRODUCTION

Collagen is the most predominant, abundant and major protein of connective tissue present in the animal body. It plays a crucial role in the physiological functions of tissues, bones, tendons, ligaments etc. The collagens are widely used in many pharmaceutical industries, food, healthcare, cosmetics and scaffold tissue engineering.[1,2,3] The collagen extracted from animals causes bovine spongiform encephalopathy, foot and mouth disease raised concern about the animal protein. Moreover the collagen extracted from pig was not used because of religious barrier. Hence an alternative remedy has been sparked for searching new source of collagen which is not having any social risks and religious barrier.

Among the collagen alternative, more attention was focused on invertebrates of which fish attracts more due to its socio-economic usage. Collagens from fish skin or swim bladder contains good substitute because of their safety and solubility in neutral salt solution and dilute acids.[4] Due to its good absorbing capacity fish collagen has super bioavailability over bovine or porcine types.[5] Fish industrial by-products such as skin, scales, fins and bones are rich in collagen and extraction of such collagen provides the aquaculture industry with a potential addition to its profit margin while addressing the increased pollution.

India is one of the largest country in practicing freshwater aquaculture which provides a major economic value to the farmer and provides number of job opportunities. *Channa Striatus* is a freshwater snake head fish cultured widely in India. *C. striatus* is a medicinal freshwater fish found in several Asian countries and used as medication to treat wounds, alleviate pain and boost energy. *C. striatus* extract may also have a role in other non-traditional uses such as in treating neurological diseases and in inducing regenerative potential of organs and cells[6] (Mohd Shafri and Abdul Manan, 2012).It is widely consumed in Asia-Pacific region as a rich source of protein and to induce wound healing in post-operative as well as post-delivery, especially among caesarean mothers[7] (Sabto, 1998). The haruan (*C. striatus*) based cream is effective for exfoliation dermatitis, such as psoriasis, eezema and ichthyosis[8] (Mat Jais *et al.*, 1997). The mucus of *C. striatus* exerts a strong antimicrobial activity.[9,10]

However no information on composition and biochemical properties of collagen extracted from the freshwater snakehead fish *C. striatus* has been reported. Hence the present study was aimed to extract and characterize the acid-solubilized collagen from the skin of freshwater snakehead fish *C. striatus*.

2. MATERIALS AND METHODS

2.1. Collection of fish

The healthy *C. striatus* were collected from Sirkali fish market, Nagapatinam District, Tamilnadu, India of an average weight 300 + 5.67g. The fish were kept in large aerated concrete tank containing potable tap water (pH 7.5 + 0.5). The tank were treated with disinfectant sodium hypochloride, with the concentration of 200 ppm for 1 hrs and washed three times with fresh tap water prior to the introduction of the fish in the water.

2.2. Preparation and extraction of Acid-Solubilized Collagen

The Acid-Solubilized collagen was extracted by following the method of Hema et al[11] with slight modification. All the extraction procedures were carried out at 4 ºC. The fish skin was minced and mixed with 30 volumes of 0.1N sodium hydroxide and kept stirred for 24 hours over a magnetic stirrer to remove non collagenous protein. The treated mass was strained through a coarse sieve. The process was repeated twice and the residue was washed twice with 30 volumes of chilled distilled water.

The residue was homogenized in a Polytron homogenizer with 30 volumes of 0.5M acetic acid for one minute and the same was stirred over a magnetic stirrer for 24 hours. The supernatant after centrifugation (3000 rpm, 20 min) was collected. The residue was once again extracted with acid as anove and the combined supernatant was taken as acid soluble collagen.

Crystalline sodium chloride was added to supernatant to the level of 10% and stirred for 24 hours to precipitate the collagen. The precipitate was suspended in Tris-glycine buffer (50 mM containing 0.2M NaCl, pH 7.4) and dialyzed against the same buffer for 24 hours and then centrifuged. The collagen obtained was spray dried to get fine powder.

2.3. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed by the method of Laemmli[12] with a slight modification. The Acid solubilized collagen samples were dissolved in 50 g/L SDS solution. The mixture were then heated at 90ºC for 1 hour, followed by centrifugation at 8000 rpm for 10 min to remove undissolved debris. Solubilized samples were mixed with the sample buffer with ratio of 1:1. The mixtures were loaded onto a polyacrylamide gel electrophoresis. The gels were then stained with 0.1% (w/v) Coomassie brilliant blue dissolved in water-methanol-acetic acid (50:40:10, v/v/v) for 1 hour. Gels were destained with a solution containing 30% (v/v) methanol and 10% (v/v) acetic acid for 30 min.

2.4. Amino acid analysis

The amino acids present in the Acid solubilized collagen of freshwater snakehead fish was assayed by following method of Dahlan-Daud *et al.*[12] 20µl of the collagen was hydrolyzed with 15ml of 6 Molar hydrochloric acid (HCl) in a closed test tube, shaken for 15 min and then flushed with nitrogen for 1 min prior to being put in an oven for 24 hrs at 110ºC. After cooling, 10ml of the internal standard α-amino butyric acid (AABA) was added to each

sample prior to the addition of 20µl redrying solution methanol : water : triethylamine (2:2:1; v/v/v) and 20µl derivatization reagent (methanol : triethylamine : phenylisocyanate, 7:1:1; v/v/v). The mixture was then poured into volumetric flasks and deionized water was added to a final volume of 50ml. To 15ml of the upper layer was discarded; the rest of the upper layer was filtered through Whatmann No.1 filter paper. The hydrolyzed sample obtained after filtration was kept for 4 weeks at -20º C until use.

Before injection into HPLC, the hydrolyzed samples were filtered using a nylon 0.2µm cellulose nitrate membrane filter. 10µl of filtered sample was put into a vial and the same volume of standard was added before the sample was dried under a vacuum for 30min. The re-drying solution (20µl) was then added to the dried sample and the mixture was shaken vigorously for 15 minutes. The sample was dried again under vacuum for another 30 minutes, followed by the addition of 20µl derivatization reagent. The mixture was shaken vigorously for 15 minutes and then left at room temperature for 20 minutes before being dried again under vacuum for 30 minutes. The dried sample was kept at -20°C until analysis by HPLC.

Prior injection to the HPLC, sample and standard were mixed with 100µl sample dilutants (Khan *et al.*, 1994), shaken for 15 minutes and injected onto the HPLC in volumes of 20 and 8 µl, respectively. The free amino acids were separated by using HPLC systems (Lachrome Hitachi). The samples were eluted with a gradient formed by acetonitrite: 25Mm potassium phosphate, pH 3.3 B=80: 20, CAN: 25Mm potassium phosphate, pH 3.3. The amino acids were eluted at the flow rate of 1 ml/min for 90 minutes. The CRS mixed amino acid in the mobile phase at the concentration of 1 mg/ml serve as standard. The column elutes were detected using a UV detector at 254 nm.

2.5. Fourier transform Infrared Spectroscopy (FT-IR)

The FT-IR investigation was carried with a spectrum RX-I FTIR system (Perkin Elmer model, USA).[13] The lyophilized products were diluted approximately 4-fold with Potassium Bromide (KBr) (FT-IR grade) and make into a pellet. The pellet was immediately put into the sample holder and FT-IR spectra were recorded in the range of 4000-400 cm^{-1}.

2.6 Measurement of zeta [ζ] Potential

The protein sample was measured using a Zetasizer Nano ZS equipped with a 2 mW helium neon laser with an output of 633 nm. Protein solutions were prepared in acetic acid (0.5 M) to a final concentration of 0.5 mg/ml followed by stirring at 4ºC for 12 hours. The pH was

adjusted to different values with either 1.0 M nitric acid or 1.0 M KOH using an Autotitrator. The isoelectric point (pl) was estimated from pH rendering a ζ-Potential reading of zero.

2.7. Effects of pH and NaCl on Collagen solubility

The solubility of Acid-solubilized collagen extracted from the skin of fresh water snakehead fish C. *striatus* was determined in 0.5 M acetic acid at various pH levels and NaCl concentrations as described by Huang et al[15] and Zeng et al.[16] The Acid-solubilized collagen was dissolved in 0.5 M acetic acid with gentle stirring at 4ºC for 12 hours to obtain final concentrations of 3 and 6 M. Approximately 8 mL of collagen solution (3 mg/l) was transferred to a centrifuge tube and adjusted across the pH range from 1 to 10 either 6 M NaOH or 6 M HCl. The volume of sample solution was made upto 10 mL with distilled water previously adjusted to the same pH as the collagen sample solutions tested. Additionally, five mL of collagen solution (6 mg/L) in 0.5 M acetic acid were mixed with 5 mL of cold NaCl in acetic acid of various concentrations to obtain final NaCl concentrations of 0 mg/ml to 60 mg/ml. All of these prepared solutions were stirred gently for 30 min and centrifuged at 12,000 rpm for 30 min at 4ºC. The protein contents in the supernatant were determined by the method previously described by Lowry et al.

3. RESULTS AND DISCUSSION

3.1. SDS-PAGE

Fig 1 shows the molecular weight pattern of the Acid –solubilized collagen against the higher molecular weight marker. The collagen extracted from C. striatus consisted of α chains (both α1 and α2 chains), β and γ chains. The α chain have a molecular weight ranges between 104 to 116 kDa. These patterns of chain was similar to the type -1 collagen which contains two identical α1 and α2 chains.[17,18] In the collagen molecule, two terminal ends are non-helical parts, which play an important role in the cross-linked structure with low solubility.[16] Similar results were obtained in the present study that the collagen extracted from the C. *striatus* has the cross linked component β-chain with a molecular weight of 200 kDa. The SDS-PAGE pattern of Acid-solubilized collagen of C.*striatus* was essentially similar to that of Nile perch[19], large fin long barbel catfish[20], brown backed toadfish[21], Ocellate puffer fish[22] and Rohu.[11]

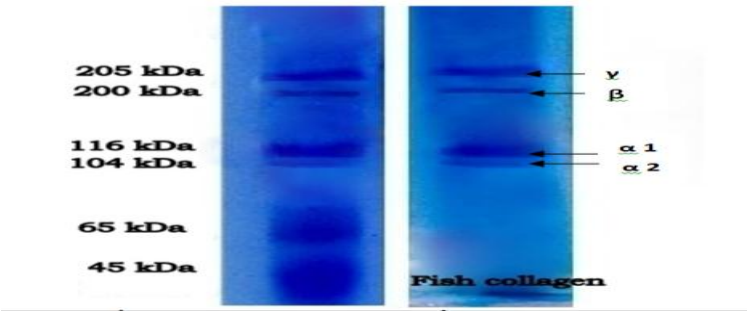

Fig. 1. SDS-PAGE of fish collagen.

3.2. Amino acid composition: The amino acid composition of Acid-solubilized collagen of freshwater snakehead fish C. striatus is shown in Table – 1 compared with the amino acid content of acid solubilized collagen of Rohu. The composition of amino acid in collagen was expressed as the number of residues per 1000 amino acid residues. Amino acid analysis showed a higher content of glycine, followed by alanine. In general glycine occurs more throughout most of the collagen extracted. Tryptophan and cysteine are not present in collagen of C. striatus. Like other collagens.[23,24] The amount of methionine, tyrosine, and histidine was very low in C. striatus. The number of proline and hydroxyproline is 178/1000 residues, which is lower than those of pig skin collagen (220/1000) and calf skin collagen (215/1000 residues).[16] The higher amino acid content plays an important role in stabilizing helices resulting in a strengthened triple helical structure to type-I collagen.

Table. 1. Amino acid composition of Acid solubilized collagen of *C. striatus*.

Sl. No	Amino acid	ASC of *C. striatus*	ASC of Rohu[11]
1	Alanine	132	130
2	Arginine	51	53
3	Aspartate	42	43
4	Cysteine	0	0
5	Glutamine	63	62
6	Glycine	331	328
7	Isoleucine	7	8
8	Leucine	20	22
9	Lysine	22	24
10	Histidine	8	7
11	Hydroxyproline	64	66
12	Methionine	10	11
13	Phenylalanine	18	18
14	Proline	114	115
15	Serine	41	41
16	Threonine	21	22
17	Tyrosine	2	1
18	Valine	25	29

3.3. FT-IR Spectroscopy

The FT-IR spectra of Acid solublizied collagen of C. striatus was shown in fig 2. The spectra clearly showed the presence of all the three major amide bands in the collagen of C. striatus. The amide bands are in the region of 1633 -1661 cm^{-1} (Amide-I), 1548 - 1558 cm^{-1} (Amide – II) and 1200 -1258 cm^{-1} (Amide – III). These amides validate the integrity of the proteins in the collagen molecules. The FT-IR spectrum study helps to determine the secondary structure of fish collagen proteins as Amide-I, II and III band. The symmetric frequency of Amide –I confirms the presence of low amount of water in the fish collagen. The triple helical structure of collagen was confirmed by the ratio of 1.0 IR absorption intensity between 1237 cm^{-1} (Amide – III) and 1548 cm^{-1} (Amide – II) respectively. The amide – I band can be ascribed to N-H[20], where a slight shift to lower wave numbers is observed comparing to non-collagens proteins (3400 – 3440 cm^{-1}). The amide – II ranges between 1548 - 1558 cm^{-1} was primarily associated with the combination of the NH plane bend and the CN stretching vibration.[25]

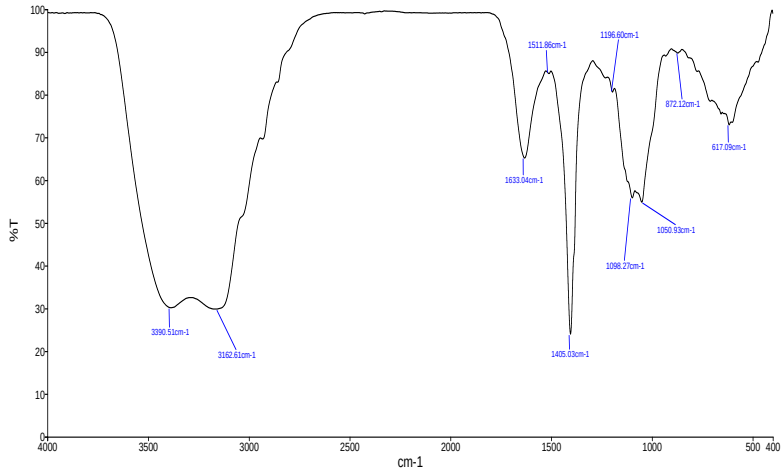

Fig. 2. FT-IR analysis of fish collagen.

3.4. ζ-Potential

The ζ-Potential of the collagen was measured as 8.01 isoelectric point at a pH of 6-9.The negative charge balanced the positive charge of the protein in an aqueous system which get zero net charge at its isoelectric point.[26]

3.5. Collagen solubility

The solubility of Acid-solubilized collagen was expressed in terms of the effects of pH (Fig 3) and NaCl concentration (Fig 4). The solubility was increased in the pH range between 2 to 4 and a dramatic decrease was occurred in the solubility ranging between pH 4 to 6. The solubility reached a minimum when the pH is raised to 8. When the pH is higher or lower the

net charge residues of protein molecules are greater and the solubility is increased by the repulsion forces between the chains. The precipitation of collagen at a particular pH may be caused by the hydrophobic interaction of the collagen molecules and the high net charge of residues in protein molecules near the isoelectric point. The solubility of collagen at different pH is in agreement with the reports of Jongia-reonrak et al[27] and Zeng et al.[16]

In the presence of NaCl the solubility of collagen was more in 10 mg/ml. when the concentration of NaCl was increased, the solubility of collagen was sharply started to decrease. This decrease was due to the impact of the salting out[28] from the fish. The NaCl concentration plays an important role in the extraction of collagen and its application.

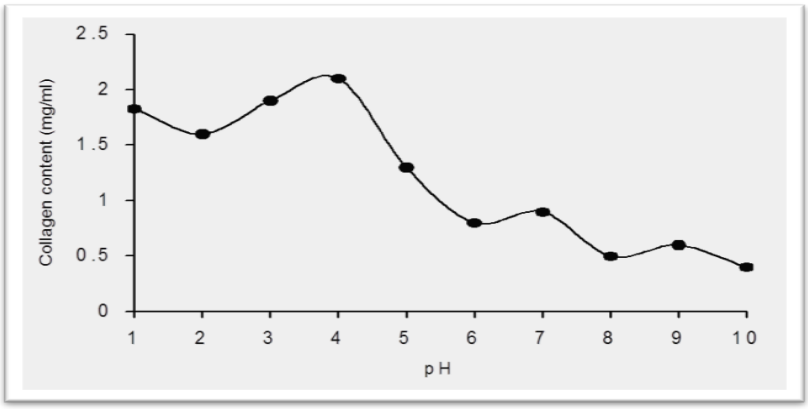

Fig. 3. Solubility of fish collagen at various Ph.

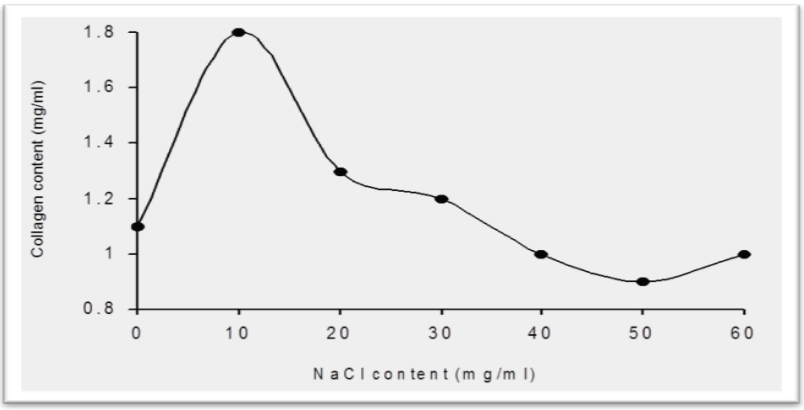

Fig. 4. Solubulity of fish collagen at different concentration of NaCl (mg/ml).

CONCLUSION

The acid-solubilized collagen from the skin of freshwater snakehead fish *C. striatus* were extracted and characterized. The result showed that collagen extracted were type-I with typical amino acid composition. The SDS-PAGE of collagen extracted from *C. striatus* has a

typical α-chains (both α1 and α2) and β-chain which confirms the triple helical structure of collagen. The FT-IR study also confirms the presence of the all the three amides with respective frequencies. The solubility of collagen was lowered with higher concentration of NaCl. These results suggest that the acid-solubilized collagen of *C. striatus* can be used as a good source of potential alternative medicine.

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